Our Reference No. 9369-153

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants:)
)
Gijs van Rooijen, Richard Glenn Keon,)
Yin Shen and Joseph Boothe)
)
Serial No. **09/643,755**)
)
Filed: **August 23, 2000**)
)
For: **Commercial Production of Chymosin**)
In Plants)

Group No.: 1638

Examiner: Georgia L. Helmer

DECLARATION UNDER 37 C.F.R. 1.132

Honourable Assistant Commissioner
For Patents
Washington, D.C. 20231

Sir:

I, Gijs van Rooijen, citizen of The Netherlands and resident of Calgary, Alberta, Canada declare that the following facts are within my knowledge and are true.

1. I am one of the named inventors of the above-referenced patent application (hereinafter "the application").

2. I am Head of Cellular and Molecular Biology at SemBioSys Genetics Inc., the owner of the application. I have been involved in research relating to plant molecular biology since 1987. I attach a copy of my curriculum vitae as Exhibit A.

3. I have reviewed the Official Action for the application that issued on September 3, 2002.

4. In particular, I note the Examiner's objection to claims 18-23 under 35 USC §112, first paragraph. In particular, the Examiner states that the application is only enabling for the isolation of chymosin from *Brassica napis* seeds. I respectfully disagree.

5. Experiments have been conducted by me or under my supervision which confirm that method of the invention can be used to produce chymosin in other plant seeds including safflower, flax and *Arabidopsis*. The experimental details are provided below.

6. **Expression and purification of chymosin from transgenic Safflower *(Carthamus tinctorius)***

Transgenic safflower seed expressing recombinant chymosin was ground in a buffer containing 250 mM NaCl and 0.5% sodium benzoate, pH 5.6. The resulting slurry was centrifuged to separate the pellet, oil body and aqueous phases. Low-pH labile proteins were removed by adjusting the aqueous fraction to pH 2, readjusting to pH 5.6 and centrifuging to remove precipitated protein. Recombinant chymosin was purified from the low-pH stable fraction through batch separation on DEAE-cellulose (Gibco Life Technologies Ltd.).

Total seed protein from a transgenic safflower line expressing chymosin was purified as described above and the different fractions were separated by SDS PAGE gel electrophoresis and stained with Coomassie Blue. As described above and shown in Figure 1 (which is attached to this Declaration as Exhibit B), the total safflower seed extract (lane 2) is ground in buffer and the aqueous fraction was adjusted to pH 2 to

remove the low-pH labile proteins (lane 3). The final step is a DEAE-cellulose purification (lane 4). It should be noted that the purity of lane 4 is comparable to the bovine derived chymosin (lane 5) and is expressed at a level of approximately 1.3% total seed protein. The expression level was determined using chymosin activity measurements, against a bovine chymosin standard curve. The results from Figure 1 represent the expression and purification of chymosin from transgenic safflower.

7. **Expression and purification of chymosin from transgenic flax (*Linum usitatissimum*)**

As shown in Figure 2 (which is attached to this Declaration as Exhibit C), 0.35 gram flax seed of transgenic line 2165-6 was ground with mortar and pestle, 10 ml of H_2O was added and the slurry was ground further (lane 2). The pH was adjusted to pH=2.23 with 1N HCl (lane 3). The slurry was centrifuged (microfuge, 10 minutes, 12000xg) and separated into pellet, oil body fraction and soluble fraction, the pH of the soluble fraction was adjusted to pH=5.6 with NaOH (lane 4). This fraction was centrifuged (microfuge, 10 minutes, 12000xg) to remove any further particulate matter, the soluble fraction obtained (lane 5). In total 1 µg of bovine chymosin (lane 1) and 5 µl of flax extract were loaded. It should be noted that lane 5 contains both the full length chymosin (A) and the chymosin C protein (B), which is a proteolytic breakdown product of the full length chymosin (Danley and Geoghegan 1988, J. Biol. Chem. 263:9785) as indicated. The results from Figure 2 show the expression and progressive purification of chymosin from transgenic flax. The expression level of the chymosin in the transgenic flax plant shown was approximately 1.15% total seed protein. The expression level was determined using chymosin activity measurements, against a bovine chymosin standard curve.

8. **Expression and purification of chymosin from transgenic *Arabidopsis thaliana***

Transgenic *Arabidopsis* plants containing the preprochymosin gene were analyzed for the presence of chymosin. 40 *Arabidopsis* seeds were ground with 50 µl 50 mM Tris HCl

pH 7.5 and added to 50 µl 2X sample loading buffer (100mM Tris pH6.8, 200mM DTT, 4%SDS, 0.2% bromophenol blue, 20% glycerol). The samples were boiled for 5 minutes, centrifuged in a microcentrifuge (5 minutes, 13,000 rpm). 15 µl of each sample was separated by SDS gel electrophoresis and stained with Coomassie Blue (Figure 3, which is attached to this Declaration as Exhibit D). For Western Blot analysis (Figure 4, which is attached to this Declaration as Exhibit E), 5 µl of each sample was separated by SDS gel electrophoresis, transferred to a membrane and probed with an anti-chymosin antibody. The results of Figure 3 and 4 show the expression of chymosin from transgenic *Arabidopsis*. Activity measurements using seeds obtained from 5 transgenic lines, generated from independent transformation events, confirmed expression levels between 1.0% and 2.3% of total seed protein (depending on the plant line selected).

9. In summary, the above results demonstrate that the method of the invention can be used to prepare at least 0.5% (w/w) chymosin in several types of plant seeds including safflower, flax and *Arabidopsis*. As a result, the application does provide an enabling disclosure of the method of the invention in any plant.

10. I declare further that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true and, further, that these statements were made with the knowledge that wilful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such a wilful false statement may jeopardize the validity of the application or any patent issuing thereon.

Dec 18, 2002
Date

Gijs van Kooijen

Exhibit A

Resume

Dr. Gijs van Rooijen
Head of Cellular and Molecular Biology
SemBioSys Genetics
110, 2985- 23 Ave NE
Calgary, Alberta
Canada T1Y 7L3
vanrooijeng@sembiosys.ca

LANGUAGES: English and Dutch (fluent written and spoken), working knowledge of German

APPOINTMENT:

Adjunct Professor (1997-2003). Department of Biological Sciences, University of Calgary

- Frequent Guest Lecturer in Botany, Biotechnology and Biology Courses and lectured on GMO regulatory issues
- Served on Graduate Students' Ph.D. committees

CAREER HISTORY:

1995 to date: SemBioSys Genetics Inc
Calgary, Alberta

1998 to date **Head Cellular and Molecular Biology**

- Responsible for 17 scientists, including 5 Ph.Ds. Responsibilities include development of scientific workplans, project planning, budgets, staffing etc. Projects include:
  - o Development of routine safflower and Arabidopsis plant transformation procedures
  - o Development of a safflower doubled haploids (funded by NRC/IRAP)
  - o Isolation of proprietary genetic elements to ensure Freedom To Operate (FTO) for transgenic applications
  - o Several other confidential proof-of concept projects for industrial clients
- Manager of a Product Development Team focused on the production and purification of a plant produced FDA regulated industrial enzyme. Launch expected June 2004
  - o Completed successful Pre-market Consultation meeting with the FDA April 2001
- Organized, chaired and a feature speaker in the "Plants as production vehicles for therapeutic proteins session at BIO2001" in SanDiego.

1995 to 1998 **Group Leader Enzymes**

- Responsible for a small technical team focused on the production of enzymes using SemBioSys' proprietary technology

1993 to 1995 **Visiting Fellow Agriculture and Agri-Food Canada Lethbridge, Alberta**

- Post Doctoral Visiting Fellow studying Pathogenesis Related genes/proteins in tobacco and potato

EDUCATION:

1989 –1993 University of Calgary
Ph.D. Specialization Plant Molecular Biology. Dissertation entitled "Molecular Biology of Oil body Proteins in the *Brassicaceae*: Structure, Function and Biotechnological applications" provided the scientific basis for the "molecular farming" applications of SemBioSys Genetics

1983 – 1988 Agricultural University, Wageningen, The Netherlands
Combined B.Sc/MSc degree Molecular Sciences

AWARDS:

Visiting Fellowship to a Canadian Government Laboratory
Nov 1993-Nov 1995
Equivalent to USDA Post-Doctoral fellowship

NSERC Post Doctoral Fellowship (Declined)
Equivalent to NSF Post-Doctoral fellowship

University of Calgary Silver Anniversary Graduate fellowship
May 1991-May 1993
Awarded to top four Doctoral students within the University (all departments)

Jake Duerksen Memorial Scholarship
May 1991-May 1993
University Fellowship

(CO) INVENTOR PATENTS :

Issued US patents

- Products for Topical Applications Comprising Oil Bodies and Antibodies US 6,372,234 (2002)
- Uses of Oil Bodies US 6,210,742 (2001)
- Uses of Oil Body Based Personal Care Products US 6,183,762 B1 (2001)
- Oil Bodies and Associated Proteins as Affinity Matrices US 5,856,452 (1999)

Pending US Patents

- Oil Bodies and Associated Proteins as Affinity Matrices: 09/707,167
- Preparation of Heterologous Proteins on Oil Bodies: 09/893,525
- Commercial Production of Chymosin in Plants: 09/643,755
- Flax Seed Specific Promoters PCT: 09/645,593, WO 01/16340 Published
- Method for Cleavage of Fusion Proteins: 09/402,488, PCT/CA/00398 Published
- Vaccines Comprising Oil Bodies: 09/880,901
- Methods for the Production of Redox Proteins and Heteromultimeric Protein Complexes, and related compositions: 10/032,201
- Thioredoxin and Thioredoxin Reductase Containing Oil Based Products: 09/897,898
- Products for Topical Applications Comprising Oil Bodies: 09/983,540

PUBLICATIONS (since 1995):

Refereed Journals

Kawchuk LM, Hachey J, Lynch D, Kulcsar F, van Rooijen G, Waterer DR, Robertson A, KokkoE, Byers B, Howard R, Fisher R, Prüfer D (2001) Tomato Ve disease resistance genes encode cell surface receptor. Proc Natl Acad Sc **98**:6511-6515

Kühnel B, Holbrook LA, Moloney MM, van Rooijen GJH*. (1996) Oil bodies of transgenic *Brassica napus* as a source of immobilized ß-glucuronidase. *J Am Oil Chem Soc* 73:1533-1538. *Corresponding author

Moloney MM, van Rooijen GJH. (1996) Recombinant proteins via oleosin partitioning. *INFORM* 7: 107-113.

Parmenter DL, Boothe JG, van Rooijen GJH, Yeung EC, Moloney MM. (1995) Production of biologically active hirudin in plant seeds. *Plant Mol Biol* 29:1167-1180.

Van Rooijen GJH, Moloney MM (1995) Structural requirements of oleosin domains for subcellular targeting to the oil-body. *Plant Phys* 109: 1353-1361.

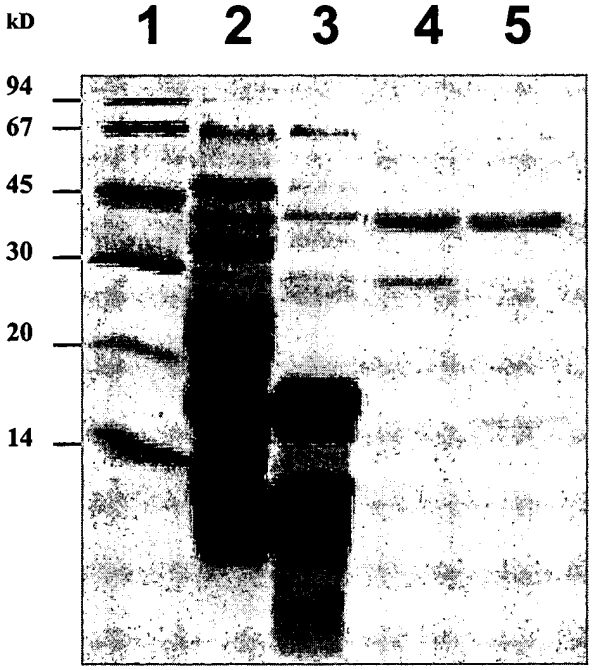
Van Rooijen GJH, Moloney MM (1995) Plant seed oil-bodies as carriers for foreign proteins. *Bio/Technology* 13:72-75.

Paper in Conference Proceedings

Van Rooijen G, Kühnel B, Kumar, Liu J-H, Mahmoud S, Moloney M. (1998) From farming for food to pharming for pharmaceuticals and other high value proteins in Crucifers (Invited paper) In: Proceedings of the International Symposium on Brassicas (Thomas G, Monteiro AA ed) p 429-443

Exhibit B

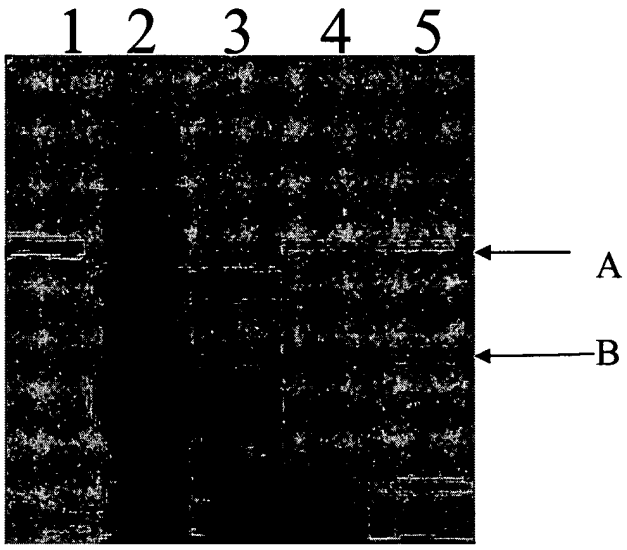
FIGURE 1



Expression and purification of chymosin from a transgenic safflower seed extract. Coomassie Blue stained gel of total seed protein from a transgenic safflower line expressing chymosin controlled by the phaseolin promoter/terminator. 1=Molecular weight markers, 2=total safflower seed extract, 3=total safflower seed extract after pH=2 treatment, 4= chymosin recovered after DEAE-cellulose supernatant of 3 after centrifugation, 5= bovine derived chymosin.

Exhibit C

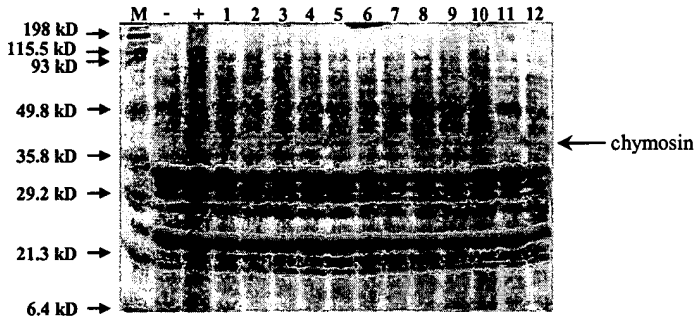
FIGURE 2



Expression and purification of chymosin from a transgenic flax seed extract. Lane 1= 1 μg of bovine chymosin, lane 2 = total flax seed extract, lane 3 = total flax seed extract after pH adjustment to pH 2.23, lane 4 = pH 2.23 adjusted fraction readjusted to pH 5.6, lane 5 = supernatant of the centrifuged pH 5.6 fraction.

Exhibit D

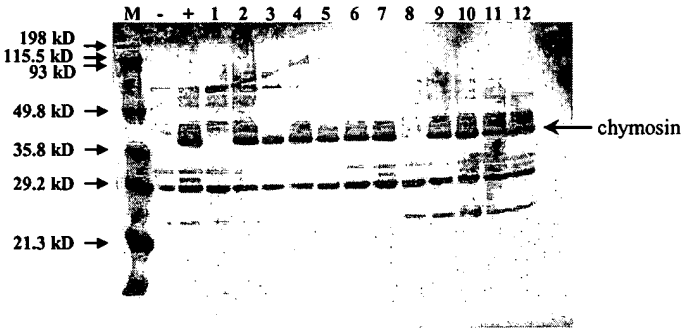
FIGURE 3



Chymosin expression in seed from *Arabidopsis thaliana*. Coomassie Blue stained gel of total seed protein. M = marker, "-" = non-transgenic control, "+" = known *Arabidopsis thaliana* seed expressing chymosin, 1-12 = independent *Arabidopsis thaliana* transformation events (T2 seeds) expressing chymosin. Chymosin is indicated by an arrow.

Exhibit E

FIGURE 4



Chymosin expression in seed from *Arabidopsis thaliana*. Western blot analysis of total seed protein. Western blot probed with an anti-chymosin antibody. M = marker, "-" = non-transgenic control, "+" = known *Arabidopsis thaliana* seed expressing chymosin, 1-12 = independent *Arabidopsis thaliana* transformation events (T2 seeds) expressing chymosin. Chymosin is indicated by an arrow.